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Form PTO-1390 (Rev. 1-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

Attorney's Docket Number	U.S. Application No. (if known, see 37 CFR 1.5)
46309-253995 (23890)	09/787016

International Application No.	International Filing Date	Priority Date Claimed
PCT/GB99/03019	10 September 1999 (10.09.99)	10 September 1998 (10.09.98)

Title of Invention
New Gene

Applicant(s) for DO/EO/US
ALONSO, Carlos Martinez; DOMINGO, David Garcia; GRANDIEN, Alf; LEONARDO, Esther; MARTINEZ, Pedro

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Transmittal of Sequence Listing (paper copy and computer readable diskette)

U.S. Application No. (if known, see 37 CFR 1.5)	International Application No.	Attorney's Docket Number
09/787016	PCT/GB99/03019	46309-253995 (23890)

17. ☒ The following fees are submitted: CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $970.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $840.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $760.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $670.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $96.00

ENTER APPROPRIATE BASIC FEE AMOUNT =				$840	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$130	
Claims	Number Filed	Number Extra	Rate		
Total claims	0 - 20 =	0	x 18.00	$	
Independent Claims	0 - 3 =	0	x 78.00	$	
Multiple Dependent Claims (if applicable)			+ 260.00	$	
TOTAL OF ABOVE CALCULATIONS =				$970	
Reduction of 1/2 for filing by small entity, if applicable. Applicant claims small entity status. -				$	
SUBTOTAL =				$970	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$970	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$970	
				Amount to be refunded:	$
				charged:	$

a. ☒ A check in the amount of $970 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 11-0855 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☐ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 11-0855. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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Patents

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	**Alonso et al.**
Application No.	**09/787,016**
I.A. Filing Date:	**10 September 1999**
Priority Date:	**10 Septemeber 1998**
For:	**Genes Encoding for the Human and Murine Death Inducer-Obliterator-1**

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Applicants herewith file a Preliminary Amendment. Applicants respectfully request consideration of the above-identified application in view of the following amendments.

Please cancel Claims 1-30 and add new Claims 31-61:

31. An isolated DNA comprising SEQ ID NO 1 and variants and alleles thereof that codes for expression of the human Death Inducer-Obliterator 1 (DIO-1) Gene.

32. The isolated DNA of Claim 31, wherein the DNA sequence is SEQ ID NO 1.

33. An isolated DNA the nucleotide sequence comprising SEQ ID NO 3 and variants and alleles thereof that codes for expression of the murine Death Inducer-Obliterator 1 DIO-1 Gene.

I hereby certify that this correspondence is being deposited with the United States Postal Service addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231 on this 27th day of August, 2001.

Mary Anthony Merchant, Ph.D. - Reg. No. 39,771

34. The isolated DNA of Claim 33, wherein the DNA sequence is SEQ ID NO 3.

35. The isolated DNA of Claim 31, comprising a fragment comprising an N-terminal domain, a central non-canonical Zn finder domain, and a C-terminus domain containing a K-rich region.

36. The isolated DNA of Claim33, comprising a fragment comprising an N-terminal domain, a central non-canonical Zn finder domain. and a C-terminus domain containing a K-rich region.

37. An isolated DIO-1 polypeptide coded for by SEQ ID NO 1 and variants and alleles thereof.

38. The polypeptide of Claim 37, comprising the mature human amino acid sequence of SEQ ID 2 and variants thereof.

39. An isolated DIO-1 polypeptide derived from the DNA SEQ ID NO 3 and variants and alleles thereof.

40. A polypeptide according to Claim 39, comprising, the mature murine amino acid sequence in SEQ ID NO 4.

41.A nucleic acid probe for the detection of a nucleic acid sequence encoding a polypeptide of SEQ ID NO 2 or SEQ ID NO 4.

42. The nucleic acid probe of Claim 41, wherein said probe comprises at least 14 contiguous nucleotides of SEQ ID NO 1 or SEQ ID NO 3.

43The isolated DNA of SEQ ID NO 1, or SEQ ID NO 3, wherein the isolated DNA comprises a cDNA sequence.

44.An expression vector containing a DNA sequence of SEQ ID NO 1, or SEQ ID NO 3, variants, alleles and fragments thereof.

45. A cell transformed with a sequence of SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, or SEQ ID NO 4, such that it allows the direct replication and expression of said sequence.

46. The cell of Claim 45 wherein the cell is a mammalian or a bacterial cell

47. A process for producing a protein encoding SEQ ID NO 2, or SEQ ID NO 4 and alleles and variants thereof, comprising culturing a cell of claim 45 in a suitable culture medium and isolating the protein thereof.

48. The process of Claim 47 wherein the cell is a mammalian or a bacterial cell.

49. A method for identifying clones encoding a DIO-1 polypeptide of SEQ ID NO 2, or SEQ ID No 4, comprising screening a genomic or cDNA library with a nucleic acid probe

according to claim 10 under low stringency hybridization conditions, and identifying those clones which display a substantial degree of hybridization to said probe.

50. A method of identifying agonists and antagonists of the protein of SEQ ID NO 2, or SEQ ID NO 4, comprising transduction or transfection of a mammalian cell line with an expression vector comprising nucleic acid sequences lacking the nuclear localization sequences or lacking the Zn finger domain or lacking the acidic domain or lacking the lysine-rich domain and thereafter identifying the agonist or antagonist interacting with the DIO-1 gene.

51. An agonist or antagonist according to Claim 50.

52. A method of identifying ligands with which the polypeptide of SEQ ID NO 2, or SEQ ID NO 4 interacts following cloning into and expression in appropriate vectors and using the two-hybrid method.

53. A method to produce specific monoclonal and polyclonal antibodies against the polypeptide according to claims SEQ ID NO 2, or SEQ ID NO 4 comprising the infection of the polypeptide to a mammalian.

54. Method for treatment of diseases which are characterized by the alteration in cell death or by hyperproliferation characterized by the administration of compounds according to SEQ ID NO 2,or SEQ ID NO 4, agonists or antagonists to SEQ ID NO 2, or SEQ ID NO 4 .

55. Method according to Claim 54 by administration of a therapeutically effective amount of the compound.

56. Method according to Claim 54 in which the disease is cancer, an auto immune disease, diabetes, rheumatoid arthritis, benign and malignant tumors or hyperproliferative skin disorders

57. Method for treatment of diseases which are characterized in the alteration in cell death or by hyperproliferation comprising introducing into a mammal a nucleic acid vector according to Claim 44 and wherein said nucleic acid vector is capable of transforming a cell in vivo and expressing said polypeptide in said transformed cell.

58. A pharmaceutical formulation comprising compounds of SEQ ID NO 2, or SEQ ID NO 4, agonists or antagonists to SEQ ID NO 2, or SEQ ID NO 4 and one or more therapeutically acceptable excipients.

59. A method for identifying a ligand to the compound according to SEQ ID NO 2, or SEQ ID NO 4, agonists or antagonists to SEQ ID NO 2, or SEQ ID NO 4 by a cell-based reporter assay. transgenic-animal reporter assay or *in vitro*-binding assay.

60. A method for identifying a substance for treatment of a condition allocated by a polypeptide of SEQ ID NO 2, or SEQ ID NO 4 comprising screening for an agonist or an antagonist of the polypeptide signal transduction to be used for treating metabolic, proliferative or inflammatory conditions.

61. A compound according to SEQ ID NO 2, or SEQ ID NO 4 or agonists or antagonists to them for use as a medicament.

In light of the amendments, Applicants are of the opinion that the application is now in condition for allowance. Such action is respectfully requested. If the Examiner believes any informalities remain in the application which may be corrected by Examiner's Amendment, or there are any other issues that can be resolved by telephone interview a telephone call to the undersigned attorney at (404) 815-6500 is respectfully solicited.

Respectfully submitted,

KILPATRICK STOCKTON, LLP

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GENES ENCODING FOR THE HUMAN AND MURINE DEATH INDUCER-OBLITERATOR-1

The present invention relates to a novel DNA sequence that codes for expression of a human Death Inducer-Obliterator 1 (DIO-1)gene and the polypeptide derived from the DNA sequence. Expression vectors containing such sequences and host cells transformed with such expression vectors are also disclosed, as are methods for the expression of the novel DIO-1 polypeptide of the invention, and uses thereof.

Background

The binding of FasL or TNF to their specific receptors triggers oligomerization and activation of a series of events that results in apoptosis (Nagata, 1997). Dissection of the signal-transducing machinery for Fas-mediated apoptosis has revealed the presence of a set of molecules, FADD/Mort1, which is recruited by and associates with Fas following its activation. In a similar fashion, experiments with cells in which apoptosis was triggered by TNF-α show the existence of another protein, TRADD, which associates to TNFR1 and triggers cell death. TRADD binds to FADD through its death domain such that both stimuli, Fas and TNF-α activate the same downstream caspase pathway. TNF-α activates still another apoptosis pathway through the recruitment of RIP, a serine/threonine kinase that activates the apoptotic pathway by yet unknown mechanisms. More recently, it has been clearly established that STAT1 is required for TNF-α -triggered cell death (Kumar *et al.*, 1997; Hoey, 1997).

Three death factors, namely TNF, FasL and TRAIL (Pan *et al.*, 1997; Gura, 1997), as well as four death receptors, Fas, TNFR1, DR3/Wsl-1 and CAR 1 (Walczak *et al.*, 1997; Chinnaiyan *et al.*, 1996), have to date been shown to play a role in apoptosis triggering. Loss-of-function mutations in the Fas system illustrate the relevance of this death factor system in maintaining lymphocyte homeostasis. The binding of these factors to their specific receptors triggers a cascade of specific cysteil proteases, the caspases, which cleave various cellular components and lead to the morphological changes characteristic of apoptosis in cells and nuclei. The known signaling pathway initiates at the cell surface and operates in the cytoplasm, the main location of the caspases as well as their inhibitors (Vucic *et al.*, 1997; Irmler *et al.*, 1997; Ghayur *et al.*, 1997; Vaux, 1997; Chinnaiyan *et al.*, 1997). Very little is known

as to how these signals are transmitted to the nucleus. A caspase-activated deoxyribonuclease (CAD) and its inhibitor (ICAD) have recently been identified in the cytoplasmic fraction of a mouse lymphoma cell line. Caspase pathway activation by different stimuli cleaves ICAD, allowing CAD to enter the nucleus and degrade chromosomal DNA (Enari *et al.*, 1998; Sakahira *et al.*, 1998).

The invention

The invention relates to the DNA sequence, amino acid sequences and compounds and methods as defined in the claims.

It also relates to the use of the new gene as defined in the claims.

The new gene has been called DIO-1 gene by the inventors.

The terms "variants" and "alleles" mean that they are derived from the sequences given in the figures and have the same function as those.

For the purpose of the invention, gene means both genomic DNA, cDNA, and synthetic DNA.

The claimed nucleotide and amino acid sequences are new. They have been found to be useful for control of apoptosis and thereby useful not only for the treatment of diseases which are characterized in the alteration in cell death or by hyperproliferation, but also for the treatment of metabolic, proliferative or inflammatory conditions.

As examples cancer, autoimmune diseases, diabetes, rheumatoid artritis, benign and malignant tumors and hyperproliferative skin disorders can be mentioned.

Figure legends

Figures 1 A, B, C and D and E. Nucleotide and predicted amino acid sequences of DIO-1. The bipartite NLS sequence is boxed and the zinc finger motifs are underlined.

Figure 1 E. Schematic representation of the predicted murine DIO-1 ORF. The starting and ending positions of the amino acids defining the motifs are numbered on top.

Figures 2. a - 2e. Northern blots and Western blot analysis, cell death and DIO-1 expression analysis.

Figures 3 a - 3c. DIO-1 expression pattern during murine limb development

Figures 4A- 4F. Overexpression of DIO-1 in chick limb.

Figure 5A - 5D. Expression pattern of several transcription factors in DIO-1-infected chick limb bud.

Detailed description of the invention

Methods

Cloning of DIO-1.

Differential display experiments were carried out using an RNAmap kit (GenHunter Corp.,) according to the manufacturer's specifications. Briefly, 200 ng of total cytoplasmic RNA (after DNase treatment with the MessageClean Kit; GenHunter) isolated from WOL-1 cells at 0, 2, 4 and 8 h after IL-7 withdrawal were reverse-transcribed with oligo(dT) primers ($T_{12}MN$) in the presence of Moloney murine leukemia virus reverse transcriptase. They were then amplified with several combinations of 5' decamer arbitrary primers and the $T_{12}MN$ used for RT in the presence of ^{35}S-dATP (1200 Ci/mmol). The amplified products were resolved in an 8 M urea, 6% polyacrylamide DNA sequencing gel and analyzed by autoradiography. Several bands of interest were isolated, reamplified, cloned in the pCR-Script SK(+) vector (Stratagene, La Jolla, CA) and further used for Northern analysis and sequencing. The full-length DIO-1 cDNA was obtained from WOL-1 cDNA by 5' RACE using a Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA), with a 3' primer called L282 (5'-AGGTGTACCTTGTACAGCAGTGAAAC-3'). The resulting 2.6 Kbp band was excised from the gel and cloned in the TA-type vector pGEM-T (Promega, Madison, WI). The resulting clones were sequence-analyzed for orientation, and the oriented sense with respect to the T7 promoter was called DIO-1pGEM-T.

To confirm the ORF sequence obtained, a cDNA library from mouse brain cloned in λZAPII (Stratagene) was screened by probing with the RACE clone: the same probe was used to screen a human fetal kidney cDNA library (Clontech) from which the human DIO-1 homologue was cloned.

Cells and transfections.

WOL-1 cells were derived from the bone marrow of adult BALB/c mice. They are an untransformed, IL-7-dependent, stroma cell- independent pre-BI cell line, capable of reconstituting irradiated SCID mice. WOL-1 cells grow in Iscove's modified Dulbecco's medium (IMDM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 1 mM sodium pyruvate, non-essential amino acids, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 10% fetal calf serum (FCS) and IL-7 (3% supernatant from a murine IL-7-producing cell line). A20, BAF/3, and FL5.12 cell

lines were maintained in RPMI 1640 with 10% FCS as described, and the FL5.12hBcl-2 stable cell line was grown in the presence of 1 mg/ml G418. MEF(10.1)Val5MycER cells were cultured in phenol red-free Dulbecco's modified Eagle medium (DMEM) containing 10% FCS at 39°C. Where indicated, 1 μM E2 (17b-estradiol) was added to the medium to activate the MycER fusion protein after 24 h FCS starvation (Wagneer *et al.*, 1994). WOL-1, A20, BAF/3, and FL5.12 cell lines were cultured at 37°C, and all cell lines were kept in a humidified atmosphere with 5% CO_2.

Transient DNA transfection was performed by electroporation. For each transfection, 2 x 10^6 log phase cells were collected by centrifugation, resuspended in 200 μl of complete RPMI 1640 medium without FCS. After addition of 10 μg of plasmid DNA (1 mg/ml), samples were gently shaken and electroporated in a 0.4 cm electrode gap gene pulser cuvette at 960 μF and 320 V with a GenePulser apparatus (Bio-Rad, Hercules, CA). Samples were then diluted with 6 ml of the same medium supplemented with 10% FCS and incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells were analyzed for cell cycle staining by FACS at 48 h post-electroporation.

Northern blot analysis.

Total cytoplasmic RNA was prepared as described (Sambrook). RNA (10 μg) was Northern blotted using a ^{32}P-labeled DIO-1 riboprobe made by DIO-1pGEM-T digestion with *Bgl* II and *in vitro* transcribed from SP6 using the Riboprobe *in vitro* Transcription System (Promega). Hybridization was performed in 50% formamide at 65°C; washes were in 0.1X SSC + 0.1% SDS at 80°C. Blots were exposed on Kodak X-OMAT AR films at -80°C with two intensifying screens.

Antibodies and Western blotting.

A peptide was synthesized corresponding to amino acids 58-72 of murine DIO-1 with an additional N terminal cysteine (CSLRRSGRQPKRTERV); it was then coupled to maleimide-activated keyhole limpet hemocyanin and the purified conjugate injected into New Zealand White rabbits. Polyclonal antibody was affinity purified against the peptide coupled to a column. WOL-1 were IL-7 starved by washing four times in complete IMDM without FCS, then resuspended in the same volume of medium plus 10% FCS.

In situ hybridization and histology

Whole mount *in situ* hybridization was carried out as described (Wilkinson, 1993) with some minor modifications (Izpisúa Belmonte *et al.*, 1993). The DIO-1 digoxigenin probe was made by *Bgl*II digestion of the DIO-1pGEM-T and transcription from the SP6 promoter. The probe used for *Lhx-2* (700 bp) encompasses the homeobox and the second LIM domain. The remaining probes have been described elsewhere, and include *Msx-1* (Robert *et al.*, 1991), *Fgf-8* (Vogel *et al.*, 1995), *NF-κB* (Kanegae *et al.*, 1998). To visualize the cartilage, embryos were fixed in trichloroacetic acid after viral infection, stained with 0.1% alcian green and dehydrated/cleared in methyl salicylate.

Production of virus and injection protocols

Chicken embryos (either from MacIntyre Poultry, San Diego, CA, or SPAFAS, Norwich, CT) were infected with a virus containing the full length cDNA of DIO-1. Virus preparation and injections were as previously described (Morgan *et al.*, 1992). After injection, the embryos were returned to the incubator at 37°C and fixed at different time points either for *in situ* hybridization or for phenotypic analysis.

Example 1

To search for genes implicated in apoptosis, the differential display PCR technique was used (Liang and Pardee, 1992) with mRNA obtained from the WOL-1 pre-BI cell line as target. WOL-1 was derived from BALB/c adult bone marrow; it grows exponentially in the presence of IL-7 and undergoes apoptosis upon IL-7 withdrawal. Using a set of oligonucleotide primers, one specific for the polyadenylated tail and the other arbitrary in sequence, mRNA was amplified from cells in exponential growth or 2 h after IL-7 deprivation; this was followed by RNA reverse transcription and resolution on a denaturing sequencing gel. Of 82 positive bands, 10 were initially identified as undergoing either upregulation or downregulation during apoptotic death and were therefore considered candidates for further analysis. They were further amplified, sequenced and compared with known gene sequences using the NCBI BLAST program. Of these, one band (Death Inducer- Obliterator 1, DIO-1) revealed that the nucleotide sequence was a novel gene in that it showed no significant identity to any known gene or translated products in the data bases. Close inspection of the DIO-1 gene showed a stretch of nucleotides with homology to Zn finger domains, nuclear localization signals, and acidic transcriptional activating domains in the amino terminal domain. Northern blot analysis of mouse tissue using a labeled DIO-1 probe

identified a major mRNA species of 9.5 Kb (see below). The cDNA clone encoded an open reading frame of 614 amino acids (Fig. 1A). In the 3' untranslated region both TA tracts and a poly(A) tail were identified. DNA searches confirmed that this nucleotide sequence was that of a novel gene.

Nucleotide and predicted amino acid sequences of human and murine DIO-1 are shown in Figures 1A, B C and D respectively. The bipartite NLS sequence is boxed and the zinc finger motifs are underlined.

The DIO-1 protein does not belong to any typical family presently identified, and comprises an N-terminal domain, a central non-canonical Zn finger domain, and a C-terminus domain containing a K-rich region. See Figure 1F which is a schematic representation of the predicted DIO-1 ORF. The starting and ending positions of the amino acids defining the motifs are numbered on top.

Example 2

To further characterize DIO-1, its expression pattern was examined by Northern blot analysis using a DIO-1 probe of RNA samples isolated from several cell lines in exponential growth or undergoing apoptosis following triggering in different conditions. Northern blots containing 10 mg per lane of total cytoplasmic RNA from the indicated cell lines, treated with several apoptotic stimuli at different time points, were hybridized to the DIO-1 riboprobe. The blots were reprobed with an actin probe for normalization of the amounts loaded. Figure 2a shows that WOL-1 in exponential growth phase expresses low levels of DIO-1, which increase upon induction of apoptosis . Conclusion: DIO-1 is upregulated in cells deprived of IL-7, or treated with IFN-γ or with dexamethasone, but not in cells treated with etoposide, UV irradiation or in those undergoing Fas-mediated cell death

Figure 2b shows Western blot analysis of WOL-1 cells driven to apoptosis by IL-7 starvation. The cells were collected at different time points after removal of IL-7 from the culture medium; 5 x 10^5 cells were lysed with RIPA buffer and the total extract electrophoresed on an 8% PAGE-SDS gel, blotted and incubated with a affinity-purified polyclonal antibody against amino acids 58-72 that specifically recognizes DIO-1 (1:100 dilution in TBS-1% dry milk). Equivalence of protein loading was confirmed by Ponceau S staining. The position of the DIO-1 gene product is indicated in Figure 2b.

The upregulation of DIO-1 mRNA levels in cells undergoing apoptosis was thus confirmed in Western blot, using the polyclonal antibody anti-DIO-1. Figure 2b shows that in cell extracts derived from WOL-1 cells undergoing IL-7 deprivation-induced

apoptosis, but not after etoposide-induced cell death, a 67 kDa band is upregulated two hours after induction. In all cases, there is a correlation between the kinetics of cell death and the upregulation of the mRNA encoding DIO-1 or of the DIO-1 protein itself.

Example 3

The effect on cell death was initially examined following transfection of DIO-1 into several cell lines using a transient transfection assay.

The DIO-1 ORF was cloned into the pcDNA3 mammalian expression vector (Invitrogen, Inc., San Diego, CA). Both empty vector and the DIO-1 construct were transiently transfected by electroporation into A20 and BAF/3 cell lines. After 48 h expression, the cells were permeabilized and stained with propidium iodide, and cell cycle analyzed by FACS. Under the same conditions, both FL5.12 wild type and stably transfected hBcl-2 cells were transiently transfected.

In Figure 2c it is shown that transfection of a DIO-1 expression plasmid into BAF/3 cells results in a dramatic loss of cell viability at 48 h post-transfection, and cells displayed morphological alterations characteristic of apoptosis, becoming rounded, condensed and finally, dying. This effect was specific in that transfection of BAF/3 with an empty vector had no effect on cell survival. To verify these results, DIO-1 plasmids were transfected into A20 (Fig. 2c) or FL5.12 cells (Fig. 2d) In Figure 2d it is shown that hBcl-2 suppresses DIO-1-induced cell death in FL5.12 cells

Conclusion: Transient transfection with plasmids containing DIO-1 induced cell death in both cases, with kinetics similar to those observed for BAF/3. When DIO-1 was transfected in FL5.12 cells overexpressing human Bcl-2, cells were resistant to cell death, showing that Bcl-2 coexpression inhibits DIO-1 death-promoting activity. In MEF(10.1)Val5MycER cells, when plasmids encoding full-length DIO-1 were transfected, cells exhibited apoptotic morphology as assessed by 4'6'-diamidino-2-phenylindole (DAPI) staining (not shown). Furthermore, using the DIO-1-specific antibody, we find that DIO-1 is located in the cytoplasm of MEF(10.1)Val5MycER cells in exponential growth. When apoptosis is triggered by addition of E2 (17b-estradiol) (Wagneer *et al.*, 1994), DIO-1 is translocated to the nucleus in apoptotic cells (not shown). Extensive efforts to derive stable DIO-1 transfectants in these three cell lines were unsuccessful, suggesting the lethality of DIO-1 expression in these cells (not shown).

DIO-1 is thus differentially expressed under several apoptotic conditions and induces apoptosis when overexpressed.

Example 4

DIO-1 expression was analyzed in murine tissues by hybridization with the DIO-1 riboprobe of a mouse MTN Blot (Clontech). To determine DIO-1 transcript distribution, various tissues were analyzed in Northern blot. Two mRNA transcript bands corresponding to 9.5 and 5.4 kb were detected in most tissues tested, including thymus, spleen, heart, brain, lung, liver, skeletal muscle, kidney and testis. This expression pattern was confirmed with the anti-DIO-1 antibody in Western blot. DIO-1 expression was also upregulated *in vitro* in various cell lines, derived from different tissues, when undergoing apoptosis

The result is shown in Figure 2e. Molecular size markers are indicated on the left. Lanes: 1, heart, 2, brain, 3, spleen, 4, lung, 5, liver, 6, skeletal muscle, 7, kidney, 8, testis.

Example 5

The development of the vertebrate limb is an amenable system for the study of signaling pathways leading to tissue patterning, proliferation and cell death (Izpisúa-Belmonte *et al.*, 1993; Schwabe *et al.*, 1998). Limbs originate as a consequence of the differential growth of cells from the lateral plate mesoderm at specific axial levels (Summerbell *et al.*, 1973). At the tip of the limb primordium, a morphologically homogenous and rapidly proliferating group of mesenchymal cells, called the progress zone, induces the overlying ectoderm to differentiate and form a specialized structure termed the apical ectodermal ridge (AER). Subsequent limb outgrowth and maintenance of the AER requires reciprocal signaling between the ridge and the underlying mesodermal cells of the progress zone (Todt and Fallon, 1984; Morgan *et al.*, 1992; Wilkinson, 1993; Vogel *et al.*, 1996). A process that involves programmed death of mesenchymal cells is also required, and a specific gene, BMP-4, has been implicated in this process. DIO-1 expression during mouse fetal development was tested and it was found that it is expressed during limb development.

DIO-1 expression pattern during murine limb development by whole mount *in situ* hybridization is shown in Figure 3a-c. BALB/c embryos from days 10.5 (a), 11.5 (b) and 12.5 (c) were hybridized to DIO-1 digoxigenin probe, showing expression in the postero-distal zone (a) of the limb. In (b), the pattern is clearly distal, with remarkable indentation in the nascent interdigitating spaces, while in (c), the expression is in the

interdigitating spaces, with no DIO-1 in the apical zone of the fingers, exhibiting another clear pattern in two areas of the antero-posterior axis.

As shown in Figure 3a-c, DIO-1 is highly expressed at gestation day 12.5 in the interdigitating membranes, where programmed cell death is known to occur.

Example 6

Cell growth, cell differentiation and cell death are intimately linked during the development of the vertebrate embryo; the developing limb is perhaps one of the best model systems in which to study this process (for a review, see Schwabe *et al.*, 1998). The AER is a pseudostratified epithelium located at the distal part of the developing limb bud shown to be required for limb outgrowth (Summerbell *et al.*, 1973; Todt and Fallon, 1984). Subsequent to the alteration in AER formation, limb outgrowth is arrested. To better understand the role of DIO-1, retroviral technology was used to misexpress it in the chick limb. A retroviral vector containing the *RCAS-Dio-1* construct, the DIO-1-ORF, was injected into the limb primordia of chick embryos at stages 8-12. At 60-72 h after injection, infected limb buds failed to develop a normal AER. Embryos were examined at different stages following infection.

The result is shown in Figures 4A-F in which A is whole mount preparation showing the leg of a wild type embryo. B is Alcian green staining of the same limb to visualize the normal cartilage pattern. C shows an infected embryo 6 days after injection showing extensive truncation of the distal elements of the leg. D shows same embryo after cartilage staining. Note the complete absence of elements distal to the tibia-fibula joint. E and F show the whole mount and cartilage staining of an embryo 8 days after infection with the *RCAS-Dio-1* construct.

In most cases, the truncations occur in the most distal elements (showing an absence of digits, carpals and metacarpals (Figure 4C and 4D). In others, reduction in size and malformations of the tibia and fibula are observed (Figure 4E and 4F and data not shown).

The infected limb is distorted and reduced in size, exhibiting an absence, reduction or malformation of phalanges, tarsals and metatarsals (dot). In a few cases, the fibula was reduced in size (asterisk).

Conclusion: Overexpression of DIO-1 inhibits chick limb outgrowth.

Example 7

Since misexpression of DIO-1 can perturb AER formation, it could be expected that it is preceded by changes in gene expression, both in the ectoderm and in the underlying limb bud mesoderm. *In situ* hybridization of embryos were infected with the *RCAS-*

Dio-1 construct using riboprobes for mesodermal genes involved in limb outgrowth such as *Msx-1*, *Lhx*-2 , and *NF-κB* (Kanegae *et al.*, 1998). *Msx-1* *Lhx*-2 , and *NF-κB* (show downregulation in their transcript levels, see Figure 5A, 5C and 5D, respectively. Furthermore, transcripts for ectodermal genes involved in limb outgrowth, such as *Fgf-8* , are also absent or downregulated, see Figure 5B). Note the reduced size of the infected limb buds (left limb buds in all cases). Transcripts for *Msx-1* (A), *Fgf-8* (B), *Lhx-2* (C) and *NF-κB*(D) are strongly downregulated (arrows) in the injected limb buds (compare with the normal expression pattern in the uninjected limb bud, right limb bud in all cases). Misexpression of the *RCAS-Dio-1* construct thus leads to arrest in limb outgrowth that is preceded by changes in the expression of genes involved in outgrowth of the limb. It is not known whether the misexpression of DIO-1 is directly responsible for the downregulation of ectodermal gene markers (i.e., *Fgf-8*) or if this is a consequence of the previously altered mesodermal gene expression. The combination of these results indicates that DIO-1 may regulate cell death and proliferation during limb development.

Conclusion: DIO-1 overexpression alters gene expression in the developing chick limb bud.

Discussion

Cell growth, cell differentiation and cell death signals are regulated through triggering of specific receptors, which leads to the activation of specific mediators, giving rise in turn to gene transcription activation and/or postranslational modification. Nonetheless, the mechanism through which this activation takes places has not yet been identified. Much work has been done on the mechanism activated by Fas and TNFR ligation in the triggering of apoptosis, but we know very little of the mechanism implicated in transcriptional regulation during the apoptotic process. Few of the molecules presently associated with apoptosis regulation are transcriptional regulators, among which p53 (Wagneer *et al.*, 1994), Nur77 (Chong *et al.*, 1997), the glucocorticoid receptor, STAT1 (Kumar *et al.*, 1997) and *NF-κB* (Kanegae *et al.*, 1998) are probably the only ones identified so far. Neither Fas- nor TNF-a-mediated apoptosis require gene transcription for induction; it may nonetheless play a role in these cases. We have recently shown that Nur77 is upregulated during Fas-mediated apoptosis, and that constitutive expression of Nur77 renders cells more susceptible to Fas-induced death. On the contrary, nuclear translocation of NF-κB prevents Fas- and TNF-α -triggered cell death, and constitutive expression of IκB favors cell death.

The structure and features of DIO-1 makes this gene useful for control of apoptosis.

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CLAIMS

1. An isolated DNA sequence according to Figure 1A and variants and alleles thereof that codes for expression of the human Death Inducer-Obliterator 1 (DIO-1)gene.

2. A DNA sequence according to claim 1, wherein the DNA sequence is that given in Figure 1A.

3. An isolated DNA sequence according to Figure 1B and variants and alleles thereof that codes for expression of the murine Death Inducer-Obliterator 1 (DIO-1)gene.

4. A DNA sequence according to claim 3, wherein the DNA sequence is that given in Figure 1B.

5. A fragment of an isolated DNA sequence according to any of claims 1 to 4, which comprises an N-terminal domain, a central non-canonical Zn finger domain, and a C-terminus domain containing a K-rich region.

6. An isolated DIO-1 polypeptide derived from the DNA sequence according to any of claims 1 to 2 comprising the mature human amino acid sequence shown in Figure 1C and variants thereof.

7. A polypeptide according to claim 6 comprising the mature human amino acid sequence shown in Figure 1C.

8. An isolated DIO-1 polypeptide derived from the DNA sequence according to any of claims 3 to 4 comprising the mature murine amino acid sequence shown in Figure 1D and variants thereof.

9. A polypeptide according to claim 8 comprising the mature murine amino acid sequence shown in Figure 1D.

10. A nucleic acid probe for the detection of a nucleic acid sequence encoding a polypeptide according to any of claims 6-9 in a sample.

11. A nucleic acid probe according to claim 10 wherein said probe comprises at least 14 contiguous nucleotides of the sequence given in Figure 1A or 1B.

12. A DNA sequence of any of claims 1 to 5 wherein the isolated DNA comprises a cDNA sequence.

13. An expression vector containing a DNA sequence of any of claims 1-5.

14. A cell transformed with a DNA sequence of any of claims 1-5, such that it allows the direct replication and expression of said DNA sequence.

15. A cell according to claim 14 wherein said cell is a mammalian or a bacterial cell

16. A process for producing a protein according to any of claims 6 to 9 which process comprises the culture of a cell of any of claims 14 to 15 in a suitable culture medium and the isolation of the protein therefrom.

17. A method for identifying clones encoding a DIO-1 polypeptide according to any of claims 6-9, said method comprising screening a genomic or cDNA library with a nucleic acid probe according to any of claims 10 to 11 under low stringency hybridization conditions, and identifying those clones which display a substantial degree of hybridization to said probe.

18. A method of identifying agonists and antagonists of the protein according to any of claims 6-9 comprising transduction or transfection of a mammalian cell line with an expression vector comprising nucleic acid sequences lacking the nuclear localization sequences or lacking the Zn finger domain or lacking the acidic domain or lacking the lysine-rich domain and thereafter identifying the agonist or antagonist interacting with the DIO-1 gene according to claims 6-9.

19. An agonists or antagonists according to claim 18.

20. A method of identifying ligands with which the polypeptide according to any of claims 6-9, interacts, following cloning into and expression in appropriate vectors and using the two-hybrid method.

21. A method to produce specific monoclonal and polyclonal antibodies against the polypeptide according to any of claims 6 to 9 comprising the injection of the polypeptide to a mammalian.

22. Method for treatment of diseases which are characterized by the alteration in cell death or by hyperproliferation, characterized by the administration of compounds according to any of claims 6 to 9 or 19 .

23. Method according to claim 22 by administration of a therapeutically effective amount of the compound.

24. Method according to claim 22 in which the disease is cancer, an autoimmune disease and/or diabetes.

25. Method according to claim 22 in which the disease is rheumatoid arthritis, benign and malignant tumors or hyperproliferative skin disorders.

26. Method for treatment of diseases which are characterized in the alteration in cell death or by hyperproliferation, comprising introducing into a mammal a nucleic acid vector according to claim 13 and wherein said nucleic acid vector is capable of transforming a cell *in vivo* and expressing said polypeptide in said transformed cell.

27. A pharmaceutical formulation comprising compounds according to any of claims 6 to 9 or 19 and one or more therapeutically acceptable excipients.

28. A method for identifying a ligand to the compound according to any of claims 6 to 9 or 19, by a cell-based reporter assay, transgenic-animal reporter assay or *in vitro*-binding assay.

29. A method for identifying a substance for treatment of a condition affected by a polypeptide according to any of claims 6 to 9, comprising screening for an agonist or an antagonist of the polypeptide signal transduction to be used for treating metabolic, proliferative or inflammatory conditions.

30. A compound according to any of claims 6 to 9 or 19 for use as a medicament.

FIGURE 1A

CTCGGTGGCC GTCCGCCCAC TCCGCGGCGT TCGGGGAAAT GGCTGCGAGA CCCTAGAGGC
CTGCGGAGCT TACTCCACGG GAACAGCCTC TAGATAATCT GAGTTGTTGA AAATACGAAG
CCTGTTACTC GTGAACAGTG GCTGACAACA GTGTTGTTGT GAGCCTGGCT GTCTGCTTGG
ACCCAGAGGT TTCGTCTGCC AGGGTTTTTG GTTGTATTTA GGATTTCAGG GAAAAGTGTC
CAAGCTTTCA GTGTTGGAGC AGGTATGGAC GACAAAGGCG ACCCGAGCAA TGAGGAGGCA
CCTAAGGCCA TCAAACCCAC CAGCAAAGAG TTCAGGAAAA CATGGGGTTT TCGAAGGACC
ACTATCGCCA AGCGAGAGGG CGCAGGGGAC GCGGAGGCTG ACCCACTGGA GCCGCCACCC
CCACAGCAGC AGCTGGGCCT GTCCCTGCGG CGCAGTGGGA GGCAGCCCAA GCGCACTGAG
CGCGTGGAGC AGTTCCTGAC CATTGCGCGG CGCCGCGGCA GGAGGAGCAT GCCTGTCTCC
CTGGAGGATT CTGGTGAGCC CACGTCCTGC CCCGCCACAG ACGCCGAGAC AGCCTCCGAG
GGCAGCGTGG AAAGCGCTTC TGAGACCAGA AGCGGCCCCC AGTCTGCTTC CACAGCTGTG
AAGGAACGAC CAGCCTCTTC TGAAAAGGTG AAAGGAGGGG ATGACCACGA TGACACCTCC
GATAGTGACA GCGATGGCCT GACCTTGAAA GAGCTTCAGA ATCGCCTTCG CAGGAAGCGG
GAACAGGAGC CCACTGAGAG GCCCCTGAAA GGGATCCAGA GTCGCCTGCG GAAGAAGCGC
CGGGAGGAGG GTCCCGCCGA GACTGTGGGC TCCGAGGCCA GTGACACTGT GGAGGGCGTC
CTGCCCAGTA AGCAGGAGCC CGAGAACGAT CAGGGGGTTG TGTCCCAGGC TGGGAAAGAT
GACAGAGAGA GTAAGTTGGA GGGAAAGGCG GCTCAGGACA TCAAAGATGA GGAGCCTGGA
GACTTGGGCC GACCGAAGCC TGAATGTGAG GGTTACGACC CCAACGCCCT GTATTGCATT
TGCCGCCAGC CTCACAACAA CAGGTTTATG ATTTGCTGTG ACCGCTGTGA AGAATGGTTT
CATGGCGATT GTGTGGGCAT TTCTGAGGCT CGAGGGAGGC TTTTGGAAAG GAATGGGGAA
GACTATATCT GCCCAAACTG CACCATTCTG CAAGTGCAGG ATGAGACTCA TTCAGAAACG
GCAGATCAGC AGGAAGCTAA ATGGAGACCT GGAGATGCTG ATGGCACCGA TTGTACAAGT
ATAGGAACAA TAGAGCAGAA GTCTAGCGAA GACCAAGGGA TAAAGGGTAG AATTGAGAAA
GCTGCAAATC CAAGTGGCAA GAAGAAACTC AAGATCTTCC AGCCTGTGAT AGAGGCGCCT
GGTGCCTCAA AATGTATTGG CCCCGGGTGC TGTCACGTGG CGCAGCCCGA CTCGGTGTAC
TGCAGTAATG ACTGTATCCT CAAACACGCC GCAGCGACAA TGAAGTTTCT AAGCTCAGGT
AAAGAACAGA AGCCAAAGCC TAAAGAAAAG ATGAAGATGA AGCCAGAGAA GCCCAGTCTT
CCGAAATGCG GTGCTCAGGC AGGTATTAAA ATCTCTTCTG TGCACAAGAG ACCAGCTCCA
GAAAAAAAAG AGACCACAGT GAAGAAGGCA GTGGTGGTCC CTGCGCGGAG TGAAGCACTC
GGGAAGGAAG CAGCTTGTGA GAGCAGCACG CCGTCGTGGG CGAGCGATCA CAATTACAAT
GCAGTAAAGC CAGAAAAGAC TGCTGCTCCC TCGCCGTCAC TGTTGTATAA ATGTATGTAT
CACCTAGGGG TTGGCCTCCT GGACCCCTCC CGTTCTTTCT GGATAGCCAT CCCCTGGGCC
TGTCCAGGAC TGGGAGTTGC AGCTTTGTGT TAAGCTGATC ACAGACACCG GCTGCACCAT
CAGCGGGAAG CAGAGCCCAT GTCCAGGATG CCTCCTGCTG CCCTGTGTCC ATCCCTAGTC
TGTCAGGACT TCCTGTCACT GTTTTCCAAA GCTGTAAACC TCACTGGTGA ACGTTCACCT
TAATGATTGA TTCTTTAATC TCTGTTTTCA CTCTCAGGCT CTGGTAAGTA TTTGTATTCT
CTTCATCCCA GTCTGATTGC ATAGCCACAC TGCCCGGCAC GCCACATCCA CCCCTGTCTG
CACATGAGTT GTTCTGACAA CAGCGCTGTA TACGCTTCAG TTTTTCCACA TTGTCCACGG
CCAGCACATG AAAGCATCAC TTCTTTTTTA TGTTGTGGGA ATCTTTGCAA GTTAGTGTTG
CATCTGATTT TCAGGTGTAC ATTTATTTTT GACTGGGCAG ATAGGGGATT TTTTTTTTTT
TCCATGTCCG ATTCACACGC TACACACCCA CATGAACACA TTCGAACTTC GAAGGCACAC
ACTCCTGCTT CATAGGCCCC ACGGTAAGTG AGTTCACACC TAGAACACTG TCCTGACCGC
AGGACGCGTG CCTTGGACTT GGTATTCTAC ATGTGACTGG CTTTCTTGCC CTCGTCTCTT
GAATGTTTAG ACTCTTAAGA TCATATCCTG

FIGURE 1B

tccgtggtag ctctggaaat ggctgcggga tcccggcggc cggggagctt gtttaagagg
cagtccccac tgtcctttg gttgttcgaa gctccggaat cttctcattg atgaactgtg
actgacaaca gtggggtgag gcttggccgt ctgcttgacc tggccccagg tctataattt
tatgtaggat ttccagccaa aggtttccaa gctttcagtt ttgggacagg tatggatgat
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cggcaaccaa aacgtactga gagggtagaa gagtttctta ccacggttcg gcgccgaggg
aaaaagaatg tgccggtgtc cctggaggat tccagtgagc ccacatcttc cacagtcact
gatgtggaga cagcttccga ggggagcgtt gaaagcagtt ctgagatcag aagtggccct
gtatctgact ccttagggaa agaacatcct gcctcttctg aaaaggcaaa aggaggtgaa
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cgccttcgga gaaagcgaga gcaagaacct gtggagaggt ccctgagagg cagtcagaat
cgcctgagga agaagcgcag agaggaagat tctgccgaaa ctgggagtgt ccaaataggc
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cggtgtgagg agtggttcca tggtgactgt gtgggtattt ctgaggcccg agggcggctc
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cagcctgact ctgtgtattg cagtaatgac tgcattctca aacacgcagc agctaccatg
agatttctaa gttcaggtaa agaacaaaaa acaaaaccca aggaaaaggt caagacgaag
ccagaaaagt tcagtcttcc aaaatgcagt gttcaggtgg ggattaaaat ctcttctgtg
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accctattga gtaaatgtac gtatcaccca aaggctggct tcccaggccc ctcccatcat
ctgggtggct gcctggggct gtctaggacc agagtcctgg gtgttctggt gctgatagta
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ctcttctata gaaaagtgat ttttttcatg agttagggca tttttgaaag gacaggtaga
caatttgtgt ctatttcaca tactacacac ctacataaac aggtttgaat tttgaaggtc
atctgctgct cagatcccat ggtgagtaat cgtgctgacc atacacctac agcagtattc
tcatcaaagt gagaattgtg ggtggtagct tccagactcg tagatgcccc agatttcaaa
tcagtgttta tagtttgagt aaacttttga aacccaagat tagatcactg agtttggagc
cattcttgtt tcactgctgt acaaggtaca cctttcttac tttttgtaca gtggtttcaa
gtgaattttc atctgtgttc tcatgatagt gttttgttcc atctctgtcc acccttagcc
ccagataagg gggtctcatg gcctaagctg gccttaaatt ttctgtaaag ttgggggtcc
tcctgccccc acctgtaaag agtgctgaaa cttacaggca cacacctgat gtatgctgtg
gggtgtgaac tcagagtgtt ggaccagcag tctaccagct gagctgcagt tctagccatg
gtaactgaaa ctgctgatct tcttgtcttt gtttcctttg tgctggg

FIGURE 1C

MDDKGDPSNEEAPKAIKPTSKEFRKTWGFRRTTIAKREGAGDAEADPLEPPPPQQQLGLSLR

RSGRQPKRTERVEQFLTIARRRGRRSMPVSLEDSGEPTSCPATDAETASEGSVESASETRSG

PQSASTAVKERPASSEKVKGGDDHDDTSDSDSDGLTLKELQNRLRRKREQEPTERPLKGIQS

RLRKKRREEGPAETVGSEASDTVEGVLPSKQEPENDQGVVSQAGKDDRESKLEGKAAQDIKD

EEPGDLGRPKPECEGYDPNALYCICRQPHNNRFMICCDRCEEWFHGDCVGISEARGRLLERN

GEDYICPNCTILQVQDETHSETADQQEAKWRPGDADGTDCTSIGTIEQKSSEDQGIKGRIEK

AANPSGKKKLKIFQPVIEAPGASKCIGPGCCHVAQPDSVYCSNDCILKHAAATMKFLSSGKE

QKPKPKEKMKMKPEKPSLPKCGAQAGIKISSVHKRPAPEKKETTVKKAVVVPARSEALGKEA

ACESSTPSWASDHNYNAVKPEKTAAPSPSLLYKCMYHLGVGLLDPSRSFWIAIPWACPGLGV

AALC

FIGURE 1D

MDDKGHLSNEEAPKAIKPTSKEFRKTWGFRRTTIAKREGAGDTEADPSEQQPQQHNLSLRRS

GRQPKRTERVEEFLTTVRRRGKKNVPVSLEDSSEPTSSTVTDVETASEGSVESSSEIRSGPV

SDSLGKEHPASSEKAKGGEEEEDTSDSDSDGLTLKEIQNRLRRKREQEPVERSLRGSQNRLR

KKRREEDSAETGSVQIGSAEQDRPLCKQEPEASQGPVSQSETDDIENQLEGKATQGNTEENP

REAGKPKPECEVYDPNALYCICRQPHNNRFMICCDRCEEWFHGDCVGISEARGRLLERNGED

YICPNCTILQVQDETNGSATNEQDSGCRSVGADGTDCTSIGTVEQKSGEDQGIKGRIEKAAN

PSGKKKLKIFQPVVEAPGAPKCIGPGCSSVAQPDSVYCSNDCILKHAAATMRFLSSGKEQKT

KPKEKVKTKPEKFSLPKCSVQVGIKISSVHKRLASEKRENPVKKVMLASRSETSGKEAACES

STPSWASDHNYNAVKPEKPEKPTALSPTLLSKCTYHPKAGFPGPSHHLGGCLGLSRTRVLGV

LVLIVASSSLPARSRYQDASGPQVFLPSLWSLSGWFLKSCVGLMLEAISYFSFRPW

FIGURE 1E

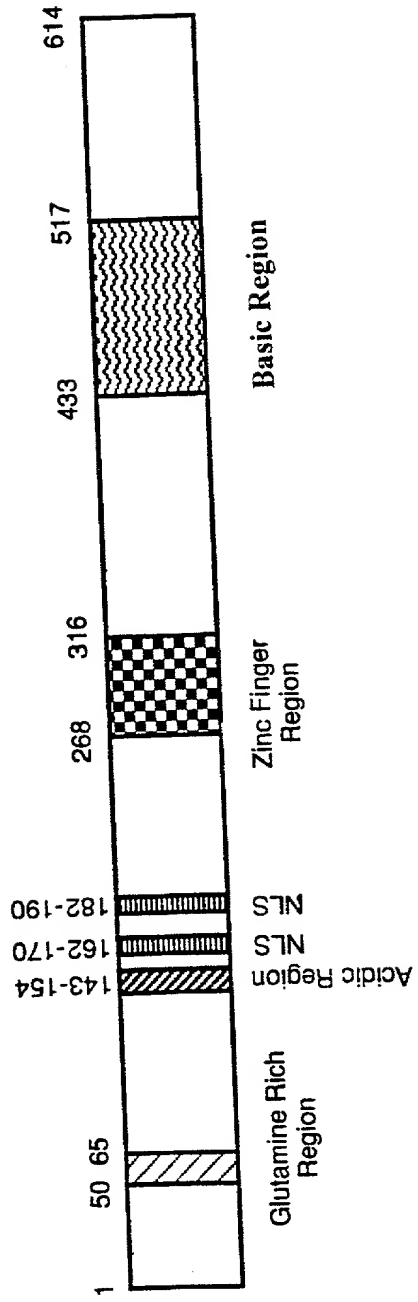

614
517
433
316
268
182-190
162-170
143-154
65
50
1
Basic Region
Zinc Finger Region
NLS
NLS
Acidic Region
Glutamine Rich Region

FIGURE 2

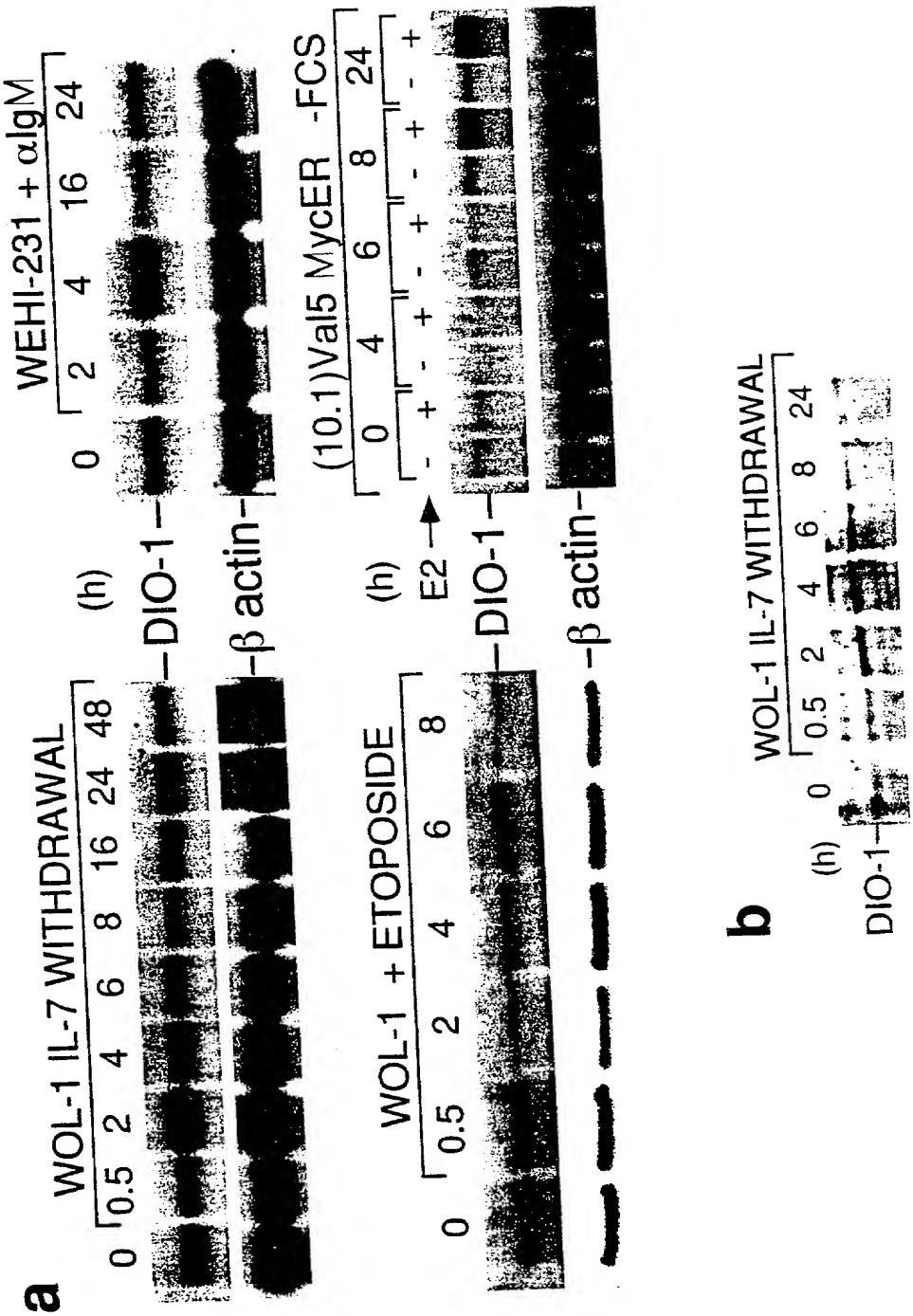

a
WOL-1 IL-7 WITHDRAWAL
(h)
0 0.5 2 4 6 8 16 24 48
DIO-1
β actin
WEHI-231 + αIgM
0 2 4 16 24
WOL-1 + ETOPOSIDE
0 0.5 2 4 6 8
(10.1)Val5 MycER -FCS
0 4 6 8 24
E2
b
WOL-1 IL-7 WITHDRAWAL
(h) 0 0.5 2 4 6 8 24
DIO-1

FIGURE 2 CONTINUED

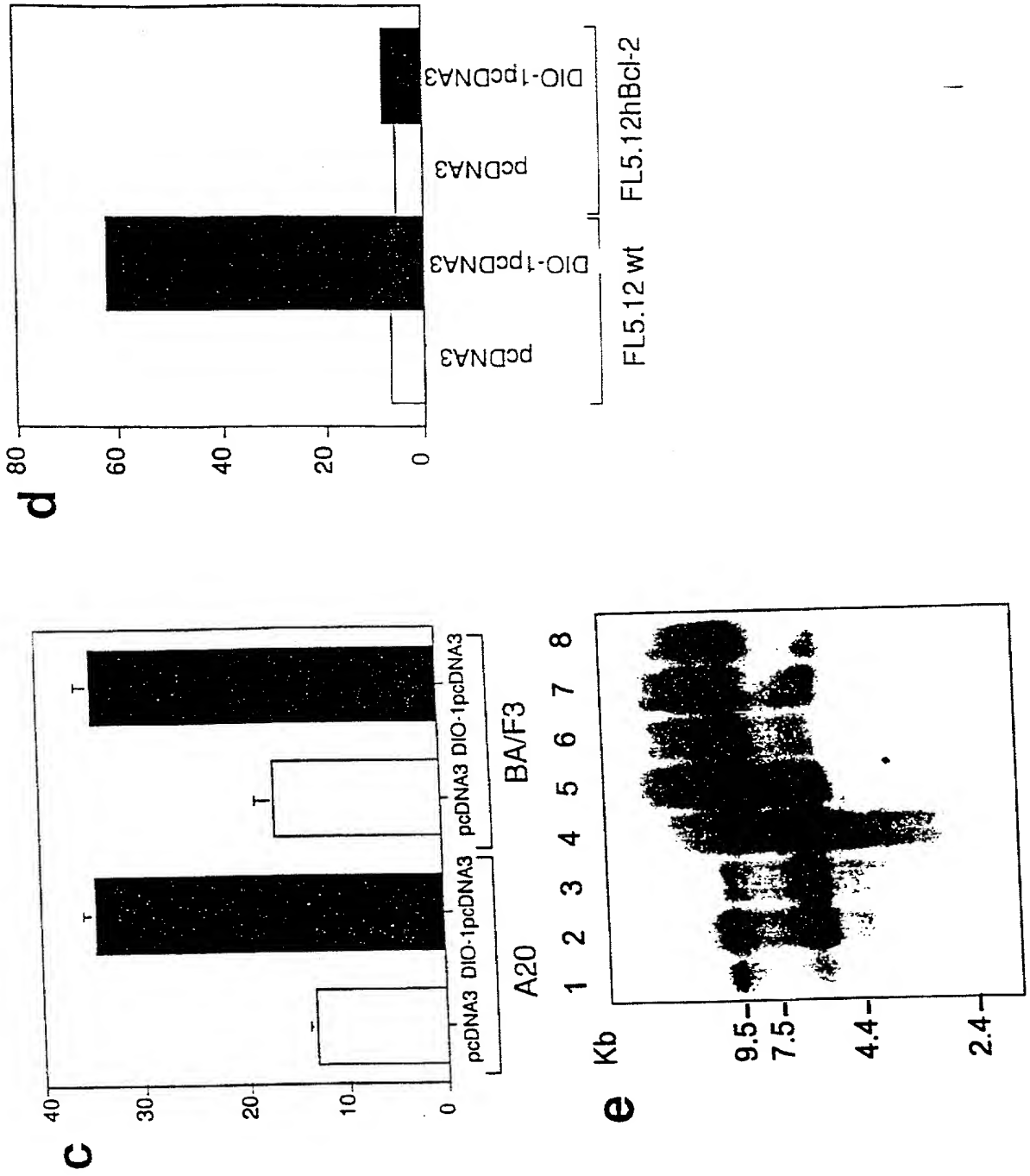


FIGURE 3

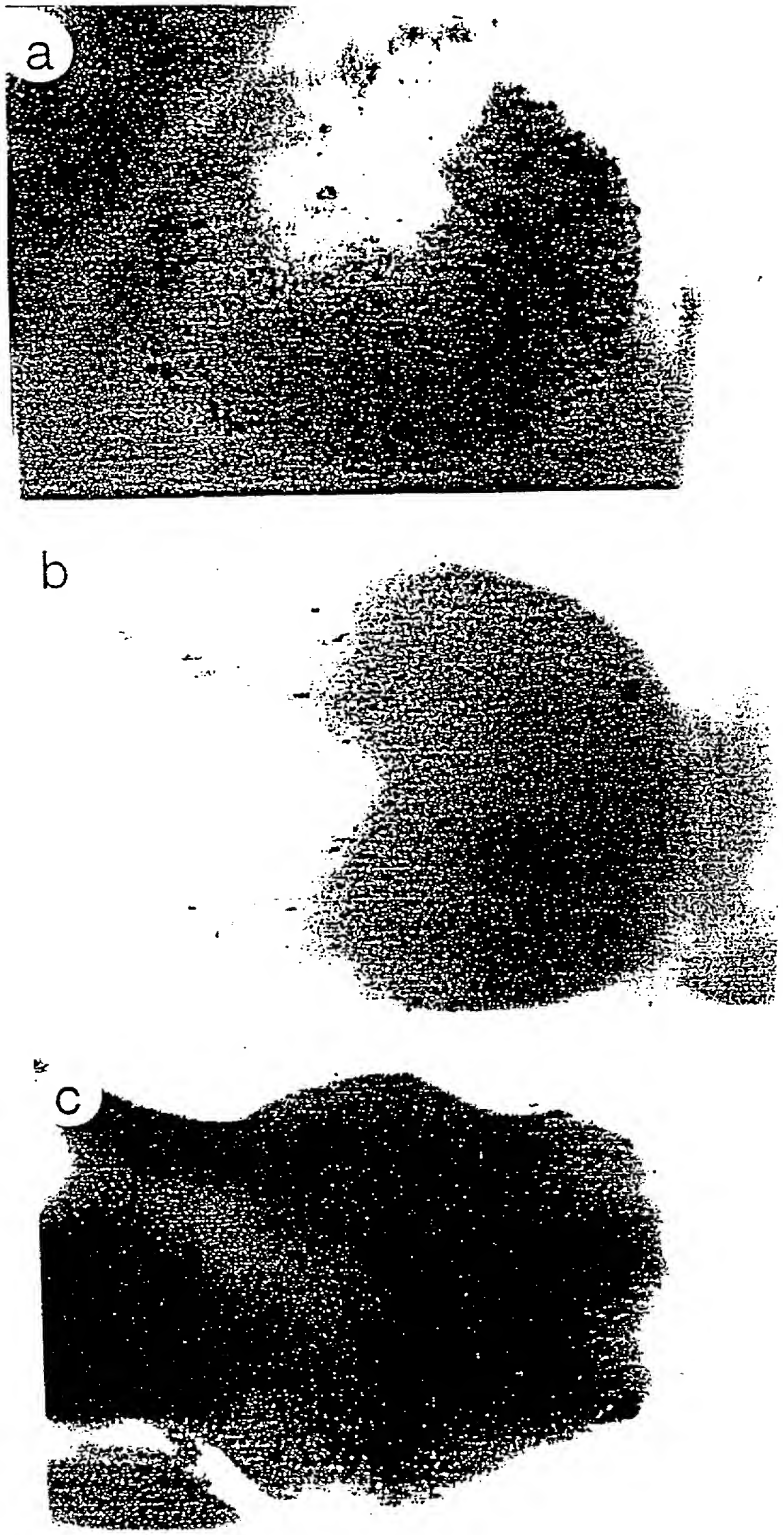


FIGURE 4

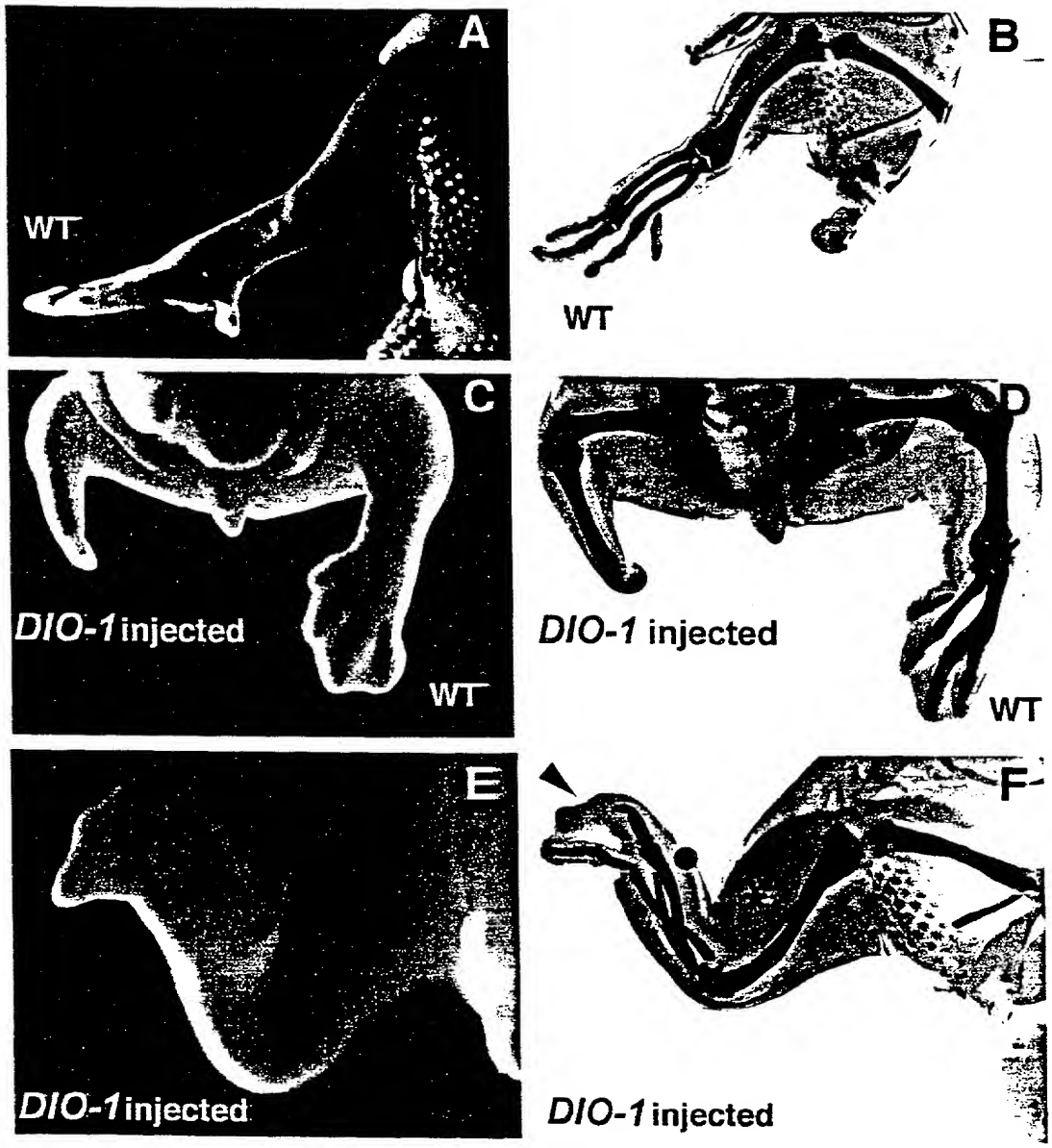
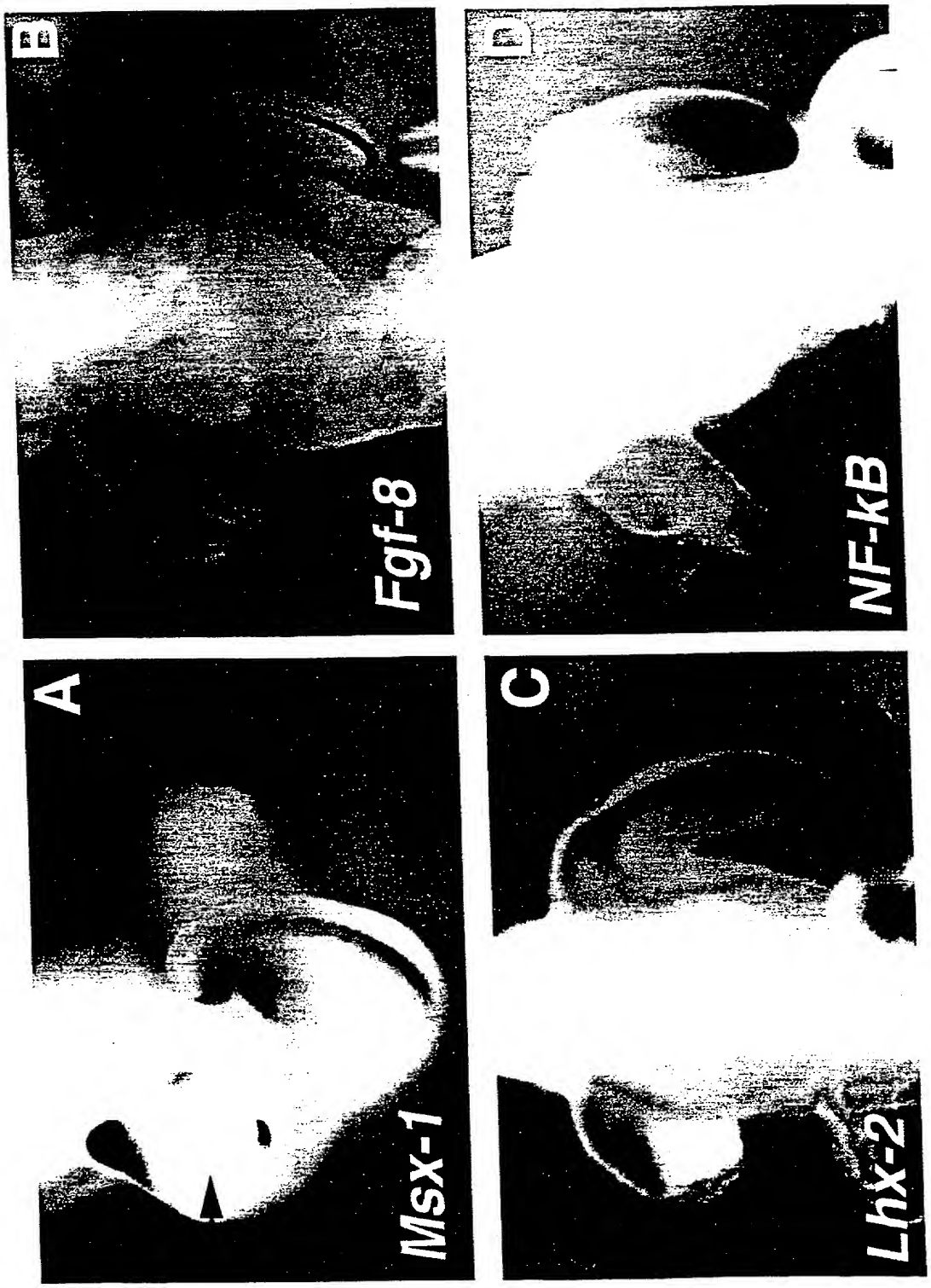

A
WT
B
WT
C
DIO-1 injected
WT
D
DIO-1 injected
WT
E
DIO-1 injected
F
DIO-1 injected

FIGURE 5



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ARATION AND POWER OF ATTORNEY

Attorney's Docket No 46309/253995

below named inventor, I hereby declare that:

residence, post office address, and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (i y one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimec l for which a patent is sought on the invention entitled: "**NEW GENE**", the specification of which

- ☐ is attached hereto.
- ☐ was filed on _____ as national phase of PCT No. PCT/GB99/03019 and was amended (if applicable) on _____.

ereby state that I have reviewed and understand the contents of the above-identificd specification, including the claims, as amended by an endment referred to above. I do not know and do not believe that the same was ever known or used by others in the United States o merica before my or our invention thereof, or patented or described in any printed publication in any country before my or our inventior ereof or more than one year prior to the date of this application. I further state that the invention was not in public use or on sale in the nited States of America more than one year prior to the date of this application. *I understand that I have a duty of candor and good faitl nward the Patent and Trademark Office,* and I acknowledge the duty to disclose information which is material to patentability as defined ir itle 37, Code of Federal Regulations, §1.56.

hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent o nventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States o America, listed below and have also identified below any foreign application for patent or inventor's certificate disclosing subject matter ir ommon with the above-identified specification and having a filing date before that of the application on which priority is claimed:

Country	App. No.	Date of Filing	Priority Claimed Under 35 USC §119
			Yes___No_____
			Yes___No_____

I hereby claim the benefit under Title 35, United States Code, § 120 of any prior United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each claim of the presen application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35 United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code o Federal Regulations §1.56, which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Filing Date	Status: patented, pending, abandoned

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statement were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

I hereby authorize the U.S. attorneys named herein to accept and follow instructions from **Withers & Rogers**, as to any action to be taken i the Patent and Trademark Office regarding this application, without direct communication between the U.S. attorney and the undersigned. I the event of a change in the persons from whom instructions may be taken, the U.S. attorney named herein will be notified by the undersigned.

POWER OF ATTORNEY: The following attorneys are hereby appointed to prosecute this application and transact all business in the Patent an Trademark Office connected therewith: **Customer Number 23594**

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☒ Additional inventors are being named on separately numbered sheets attached hereto.

y Docket No.: 46309/253995
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